Binding of p-Nitrophenyl Phosphate and Other Aromatic Compounds by β-Lactoglobulin

HAROLD M. FARRELL, JR.,[1] MICHAEL J. BEHE, and JUDITH A. ENYEART
Eastern Regional Research Center[2]
600 East Mermaid Lane
Philadelphia, PA 19118

ABSTRACT

Results obtained from gel filtration showed that β-lactoglobulin binds p-nitrophenyl phosphate with a stoichiometry of 1 mol of ligand per 18,360 monomer. Circular dichroic spectra confirmed the binding and implicated tryptophan and phenylalanine residues in the interaction. Fluorescence of the protein was quenched on binding also supporting complex formation; analysis of these data indicates that p-nitrophenyl phosphate binds to β-lactoglobulin A with a dissociation constant of 31 μM. The B and C genetic variants of β-lactoglobulin bind p-nitrophenyl phosphate with dissociation constants of 63 and 70 μM, respectively. In addition, a series of other nitrophenyl compounds and pyridoxal phosphate were also investigated by fluorescence analysis and found to bind to the protein. These results are discussed with respect to a recent hypothesis that β-lactoglobulin binds retinol and is structurally related to serum retinol binding protein.

INTRODUCTION

β-Lactoglobulin has been well-characterized for its chemical and physical properties (22), yet no biological activity had been ascribed to this molecule. Recently Pervaiz and Brew (16) and Godovac-Zimmerman et al. (9) presented evidence that strong structural homology exists between β-lactoglobulin (β-Lg)[3] and the retinol binding protein of human serum (RBP). The latter workers suggested that tryptophan 19 is conserved during evolution in both β-Lg and RBP and might represent a portion of a known aromatic binding site on β-Lg (7, 8, 17).

During a series of investigations into the mechanism of casein phosphorylation and dephosphorylation (2), β-Lg inhibited the hydrolysis of p-nitrophenyl phosphate (p-NPP) by phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) and β-Lg bound the substrate during the reaction (6). This paper reports the binding of p-NPP, other nitrophenyl derivatives, and pyridoxal phosphate by β-Lg. These interactions are discussed in relation to retinol binding by β-Lg and to the homology between β-Lg and RBP.

MATERIALS AND METHODS

The bovine β-Lg genetic variants, A, B, and C, were prepared by the method of Aschaffenburg and Drewry (1) and recrystallized 4×; the B and C variants of β-Lg were the gift of J. J. Basch of this laboratory. The sodium dodecyl sulfate derivative of β-Lg was recrystallized as previously described (19). p-Nitrophenyl sulfate, p-nitrophenyl β-glucuronide, p-nitrophenyl phosphate (Grade A), p-nitrophenol, and pyridoxal phosphate were all purchased from Calbiochem.[4] We prepared solutions by dissolving the proteins or the ligands in sodium or ammonium acetate at half the volume required; solutions were then titrated with sodium or ammonium hydroxide to pH 6.0 and diluted to concentrations of 10 or 100 μM. Concentrations of β-Lg were determined by use of the extinction coefficient .960 1 g^{-1} cm^{-1} (5) at 278 nm.

Column chromatography was carried out by the method of Hummel and Dreyer (11) using Sephadex G-10. A Pharmacia column .9 × 30 cm was equilibrated with the eluting buffer (10 mM ammonium acetate pH 6.0), which con-

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[1] To whom correspondence should be addressed.

[2] Agricultural Research Service, US Department of Agriculture.

[3] Unless specifically stated, all experiments are with the A variant.

[4] Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

tained a given concentration of p-NPP; constant flow rate was maintained by the use of a Milton Roy pump. The β-Lg was dissolved in the buffer-p-NPP solution and pumped onto the column by the use of sample tubing and three-way Luer-Lok valves. The column effluent was monitored at 310 nm on a Gilford 2000 recording spectrophotometer. Because these experiments were carried out with varying initial p-NPP concentrations (.7, .5, and .3 m*M*), the number of moles of p-NPP bound per mole of β-Lg ($\bar{\nu}$) can be obtained, and a value of dissociation constant (K_D) obtained from the equation

$$\frac{1}{\bar{\nu}} = \frac{1}{n} + \frac{K_D}{n[L]} \qquad [1]$$

where n equals the number of binding sites and [L] is the free concentration of p-NPP (20).

Circular dichroism (CD) spectra were obtained on a Durrum-Jasco ORD/UV-5 instrument equipped with a CD attachment. For spectra from 500 to 260 nm, matched 1.0-cm quartz cells were used; .1-cm cells were used below 260 nm. The CD spectrum of the sample was run and the base line determined on the blank immediately; three sample scans and three base line determinations were made on each solution. The entire experiment was then repeated twice with freshly prepared solutions, yielding a total of nine scans; with the aid of a computer program, the base line was subtracted at each nanometer and the spectra averaged. The ΔA to [Θ] conversions were carried out as previously described by Townend et al. (23).

Fluorescence emission spectra were obtained on 2-ml samples in 1-cm fluorescence cells on an Aminco Bowman spectrofluorometer equipped with a solid state microphotometer. The λ_{max} of excitation was first determined on the fluorometer (for β-Lg this corresponds to 280 nm) and the emission spectra subsequently obtained are presented uncorrected for variations in instrument output and response. For titration experiments, peak intensities were read from the recorded spectra and corrected for inner filter effects by use of K (OD) as proposed by Brand and Withold (3). The data were then analyzed (13) by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_Q [L] \qquad [2]$$

where F_0 is the relative fluorescence of the protein in the absence of added concentrations of ligand [L] and K_Q is the quenching constant. This latter term (13) contains the apparent biomolecular rate constant for quenching, k_q, and the fluorescent life time of the protein τ so that:

$$K_Q = k_q \tau \qquad [3]$$

When K_Q is taken as a measure of binding, it was used as a first estimate of the K_D,[5] and the iterative method of Yoshida et al. (24) was used to obtain a better estimate of K_D for the complex. With an assumed 1:1 stoichiometry, the K_D can be obtained from the following expression:

$$X = \tfrac{1}{2}\left[(1 + a + kd) - ((1 + a + kd)^2 - 4a)^{.5}\right] \qquad [4]$$

where a is the molar ratio of total ligand to total protein and kd is K_D divided by the total molar protein concentration. A plot of the change in fluorescence (ΔF) at λ_{max} as a function of X will give a family of curves for various estimated dissociation constants, and the closest approximation of the dissociation constant will yield a straight line. A computer program was used to obtain this dissociation constant by iterative evaluation of correlation coefficients of the lines.

Data from fluorescence were also analyzed (13) by the double reciprocal form of the Stern-Volmer equation:

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_Q [L]} + \frac{1}{f_a} \qquad [5]$$

Here F_0 and K_Q have the same meanings as in Equation [3]; ΔF is $F_0 - F$ and f_a is the fraction of initial fluorescence available to the ligand L.

[5] The K_D for the protein (P) ligand (L) interaction is defined as equal to (P) (L)/(PL).

RESULTS AND DISCUSSION

Demonstration of Complex Formation by Gel Filtration

Samples containing 1:10 molar mixtures of β-Lg and p-NPP were placed on a Sephadex G-10 columns containing 10 m*M* ammonium acetate pH 6.0. No evidence of complex formation was obtained, since no p-NPP eluted at V_0 along with the protein. Thus, complexes, if formed under these conditions, which are similar to those of Robillard and Wishnia (17), are readily dissociated. The method of Hummel and Dreyer (11), however, is capable of detecting such dissociable complexes. In these experiments, the entire column was equilibrated with the p-NPP (1 m*M*) and buffer. The β-Lg, dissolved in the p-NPP buffer mixture, was introduced as the sample. If an interaction has occurred, a trough should appear at V_{total}. This is seen to be the case in chromatogram VI (Figure 1) at the elution time of about 50 min. Excess p-NPP can be dissolved along with the protein in the eluting buffer and the trough titrated (chromatogram VI, Figure 1a, elution time 50 min) into a peak (chromatogram 1, Figure 1, elution time 50 min). When the integrated area of the peak or trough is plotted against the amount of added p-NPP (Figure 2), the binding ratio of p-NPP:β-Lg can be found where the plot crosses y = 0. This reveals a 1:1 stoichiometry for the binding of p-NPP to the β-Lg per monomer of 18,000 daltons. Although β-Lg exists as a symmetrical dimer under these conditions, it is convenient to treat the binding data as though it were a monomer. Robillard and Wishnia (17) studied the binding of toluene and perfluorinated aromatics to β-Lg. They concluded that one hydrophobic binding site exists per monomer and demonstrated that treatment of the β-Lg as though it were a monomer does not influence the K_D obtained. Repetition of these experiments at other p-NPP concentrations can give an approximate value of K_D for the p-NPP-β-Lg complex. This value is 60 μ*M*. These results demonstrate a β-Lg complex with p-NPP.

Spectral Evidence for Complex Formation

Difference spectra obtained on the Cary-14 showed no change in the absorption spectra upon combination of p-NPP and β-Lg under a variety of conditions. The CD spectra of β-Lg and p-NPP in combination showed no changes in the backbone region (210 to 260 nm) nor in the visible region (300 to 600 nm). However, significant differences in the chromophoric region (260 to 300 nm) of the CD spectra of β-Lg were detected in the presence of p-NPP (Figure 3); this indicates that the molecules are interacting in solution, as p-NPP exhibits no CD spectra of its own. Changes in the β-Lg CD spectra can be detected at 272, 275, and 283 nm. It is not unusual for CD spectra to reveal interactions that are not readily detectable by simple absorbance measurements. Tentative assignment of the CD bands of β-Lg in the near

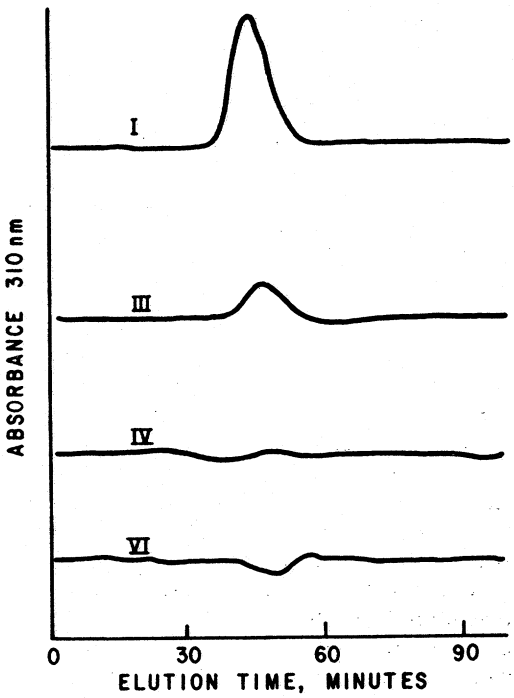

Figure 1. Sephadex G-10 chromatography according to the method of Hummel and Dreyer (13). Samples containing 40 nmol of β-lactoglobulin (β-Lg) A and varying amounts of additional p-nitrophenyl phosphate (p-NPP) were dissolved in 1 ml of the eluting buffer (10 m*M* acetate plus 1 m*M* p-NPP). Chromatograms were obtained by monitoring the absorbance at 310 nm with a full scale difflection of 1.0 absorbance unit. Roman numerals I, III, IV, and VI relate to the chromatograms obtained by adding 540, 162, 49, and 0 nmol of p-NPP along with the β-Lg; experiments were also conducted with p-NPP at 270 and 25 nmol (chromatograms II and V, not shown).

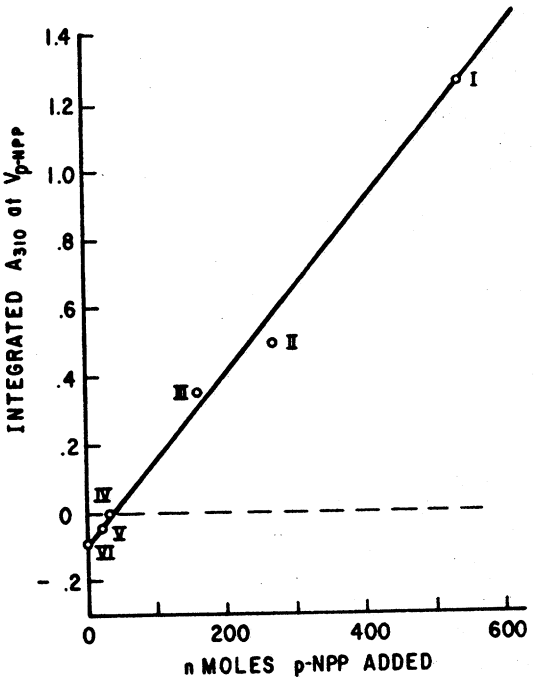

Figure 2. The integrated areas of the peaks or troughs obtained in Figure 1 for the p-nitrophenyl phosphate (p-NPP) elution volume (50 min) are plotted against the nanomoles of p-NPP added with the sample.

ultraviolet region has been made (21). Bands at 285 and 293 nm have been assigned to tryptophan, while bands at 277 and 266 nm have been assigned to phenylalanine. These experiments indicate that in the interaction of p-NPP with the protein, tryptophan, and phenylalanine may be involved. Fugate and Song (7) showed that tryptophan modification by 2-hydroxy-5-nitrobenzyl bromide altered, but did not completely inhibit, retinol binding to β-Lg, whch supports the concept of tryptophan involvement in the formation of the binding site.

On the basis of sequence homology, Godovac-Zimmerman and coworkers (9, 10) have implicated tryptophan 19 of β-Lg in the binding of retinol. It is interesting to speculate that tyrosine 20, which is directly adjacent to this tryptophan, might also be involved, but the CD bands that change may not be related to tyrosine. Tryptophan 61, however, occurs in a region of uncertain structure and is not conserved in horse β-Lg (10). In contrast, crystallographic data for RBP shows that a β-barrel serves as the retinol binding site and that no tryptophan is within 5 Å of the retinol (15), indicating a potential difference between RBP and β-Lg. Reasons for this could be based in structural differences. Creamer et al. (4) predicted, based on Chou-Fasman analyses, that β-strands of 5 to 6 residues in length occur periodically from residues 1 to 45 in β-Lg; folding of this region could easily form a β-barrel similar to that of RBP. E. M. Brown (this laboratory, personal communication) has a somewhat different analyses of residues 1 to 45, which still involves β-structure but places tryptohan 19 squarely in a β-strand, which could be part of a β-barrel. The occurrence of proline residues at positions 38 and 48 in β-Lg (10, 15) supports a change in the length of the β-sheets; change in the overall nature of the β-barrel may bring residues such as tryptophan into the site. Although examination of the 6 Å resolution structure of β-Lg (18) indicates an area where such a β-barrel might occur, the exact stereochemistry of the residues present in this region is unknown.

Fluorescence Study of the Complex and its Dissociation Constant

The fluorescence emission spectrum of β-Lg (in 10 m*M* ammonium acetate, pH 6.0) was

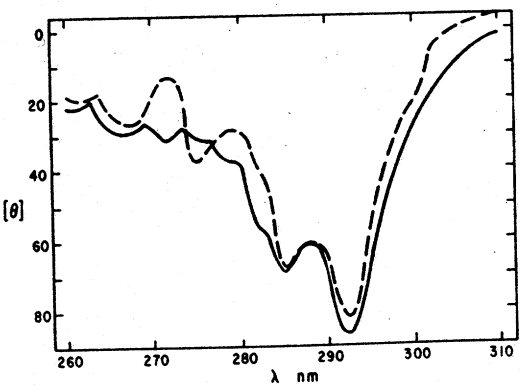

Figure 3. The circular dichroism spectra of β-lactoglobulin (β-Lg) (——), and β-Lg plus p-nitrophenyl phosphate (p-NPP) (----) at pH 6.0, 10 m*M* ammonium acetate. The β-Lg A concentration was 112 μ*M* (1.94 g/L) in both cases and the p-NPP concentration was 180 μ*M*. The ΔA_{L-R} from nine scans was averaged as described in Materials and Methods.

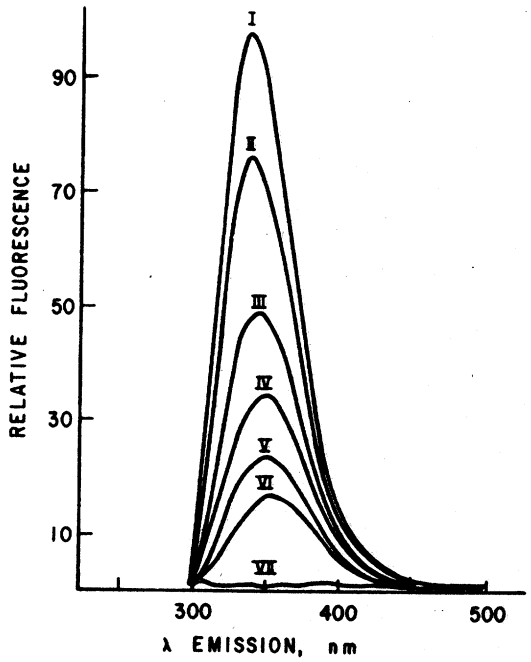

Figure 4. Effect of p-nitrophenyl phosphate (p-NPP) on the fluorescence emission spectra of 18.7 μM β-lactoglobulin (β-Lg) A. The buffer employed was 10 mM sodium acetate, pH 6.0. Spectra I β-Lg alone; spectra II through VI represent added p-NPP at 8, 32, 48, 64, and 80 μM, respectively. Spectrum VII represents p-NPP alone at 100 μM.

studied as a function of added p-NPP concentration, and p-NPP quenched the fluorescence of the β-Lg molecule (Figure 4). The quenching observed is indicative of the formation of a complex, when analyzed as follows: inner filter effects were first corrected mathematically (3), then Stern-Volmer plots of F_0/F against concentration of p-NPP were constructed (Equation [2]). These plots were linear (Figure 5) and gave an apparent quenching constant (K_Q) of 28,800 M^{-1}. Using this latter value, and the fluorescent lifetime of β-Lg of 1.4 ns (7), the apparent biomolecular rate constant can be calculated from Equation [3]. For the quenching of β-Lg by p-NPP, this value was 2×10^{13} M^{-1} s^{-1}, which exceeds any possible diffusion controlled processes that occur with maximum rate contants of 1.4×10^{11} M^{-1} s^{-1}. Thus, complex formation occurs prior to the fluorescence experiment. These results coupled with the comparable K_D obtained gel filtration clearly show a complex between β-Lg and p-NPP. Analysis of the data by Equation [5] (Figure 5, inset) yield a value of 1 for f_a, the fraction of tryptophan quenched. This demonstrates that both tryptophans 19 and 61 are either closely related or that energy transfer within the β-Lg is interrupted by the binding. Mills (14) has shown that both tryptophans are in similar hydrophobic environments, but they can be differentiated by partial denaturation.

An estimate of the K_D for the β-Lg-p-NPP complex can be obtained by iterative calculations (24) on the change in fluorescence as a function of added ligand. The K_D for p-NPP binding by three genetic variants of β-Lg [A, B, and C, see reference (5)] were determined (Table 1). β-Lactoglobulin A forms the strongest complex followed by B and C. Seibles (19) described the preparation of a crystalline sodium dodecyl sulfate complex of β-Lg, which has a stoichiometry of 1 mol of detergent/mol of protein monomer. This sodium dodecyl sulfate derivative of β-Lg A has a K_D for p-NPP identical with that of the uncomplexed protein; the dodecyl sulfate complex of β-Lg (7) also binds retinol nearly as well as the native protein. The K_D for β-Lg-p-NPP is independent of pH in the range 4.0 to 7.5; this was also found by Fugate

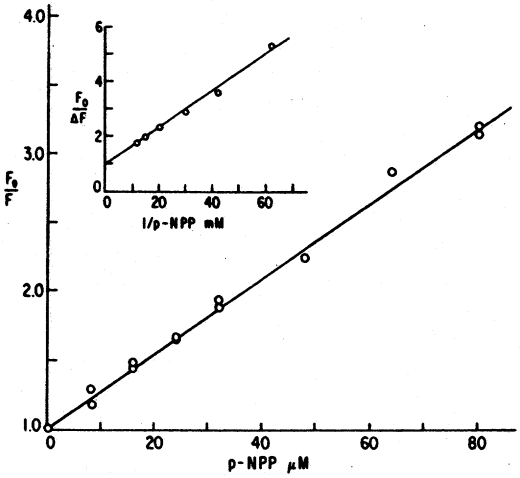

Figure 5. Stern-Volmer plot (main figure) for p-nitrophenyl phosphate (p-NPP) quenching of β-lactoglobulin (β-Lg) fluorescence according to Equation [2]; the slope is equal to K_Q. The inset shows the modified double reciprocal plot of Equation [5]; the y intercept is equal to $1/f_a$.

TABLE 1. Dissociation constants for β-lactoglobulin (β-Lg)-p-nitrophenyl phosphate (p-NPP) complexes determined by fluorescence quenching.

Ligand	Protein	K_D	
		(μM)	(σ)
10 m*M* sodium acetate			
p-NPP	β-Lg A	31	14
p-NPP	β-Lg B	63	11
p-NPP	β-Lg C	70	19
100 m*M* sodium acetate			
p-NPP	β-Lg A	45	9

[1] Average of four or more experiments (± σ, standard deviation); conditions as described in Figure 3.

TABLE 2. Dissociation constants (K_D) of some β-lactoglobulin A complexes as determined by fluorescence quenching.

Ligand	K_D[1]
	(μM)
p-Nitrophenol	52
p-Nitrophenyl acetate	33
p-Nitrophenyl β-glucuronide	64
p-Nitrophenyl sulfate	510
p-Nitrophenyl phosphate	31
Pyridoxal phosphate	320

[1] Average of three experiments; conditions as described in Figure 3.

and Song (7) for retinol binding by β-Lg. Increased salt (100 m*M*) does not appreciably alter the binding of p-NPP either (Table 1).

In addition to p-NPP, other p-nitrophenyl compounds were tested for binding to β-Lg A, and K_D calculated; results are similar (with the exception of p-nitrophenyl sulfate) and are given in Table 2. The K_D obtained in this study for nitrophenol and its derivatives are all in the range of 30 to 60 μ*M*. The K_D for p-nitrophenyl sulfate was 10 times larger (less tight binding); a similar value was found for pyridoxal phosphate, a soluble cofactor structurally analogus to p-NPP.

Robillard and Wishnia (17) estimated that the binding site of β-Lg may be limited in size

TABLE 3. Comparison of the dissociation constants (K_D) obtained for binding of aromatics by β-lactoglobulin A.

Ligand	K_D	Reference
	(μM)	
Toluene	2200	(17)
Trifluoro toluene	2390	(17)
Hexafluorobenzene	640	(17)
Pyridoxal phosphate	320	[1]
p-Nitrophenyl phosphate	31.0	[1]
Retinol	.020	(7)

[1] Values obtained in this study.

to single ring aromatics. Values for K_D obtained for single ring compounds in other studies of β-Lg are compared with those found here in Table 3. Retinol forms the tightest binding complex (Table 3) and argues for an evolutionary relatedness for β-Lg and RBP. Retinol and its derivatives, however, occur in the lipid phase, presumably surrounded by fat globule membrane (12), and are not associated with β-Lg in milk or mammary tissue. Thus, retinol binding by β-Lg maybe a vestigal property. As noted, structural changes may make the β-Lg binding site different than that of RBP and so tryptophan and phenylalanine may play more important roles in binding aromatics. The binding of aromatics other than retinol with weaker K_D may also point to other possible roles in mammary metabolism for β-Lg. Complexes with K_D, such as those of β-Lg and pyridoxal phosphate, are more apt to be able to alter enzyme activity or to affect reversible transport in mammary tissue. The possibility of β-Lg acting to regulate phosphate metabolism has been previously discussed (6).

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